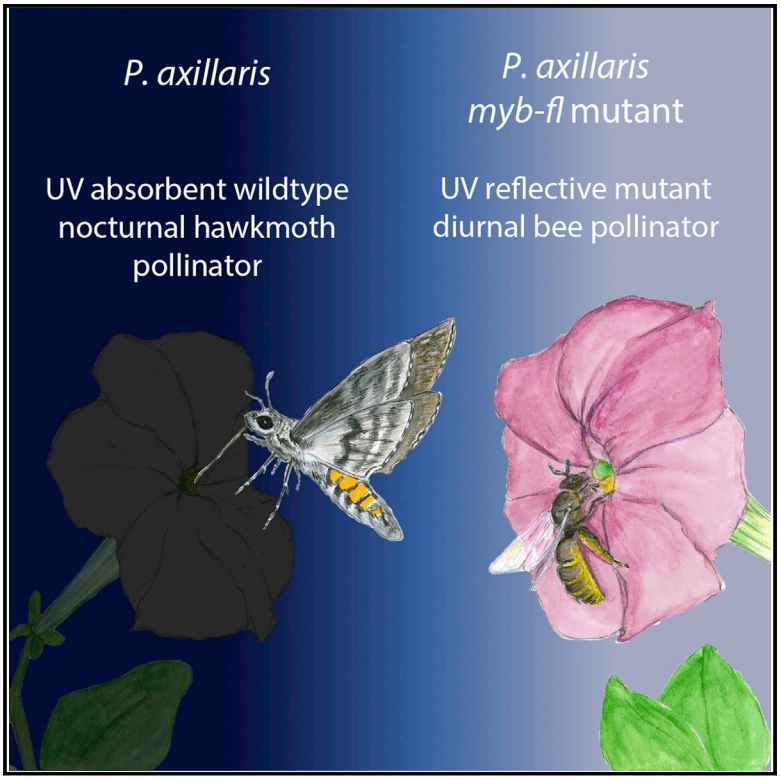
Article

Current Biology

Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference

Graphical abstract



Highlights

- *MYB-FL* mutation strongly reduces floral UV color but increases visible color
- The mutation reduces hawkmoth visitation but increases bee visitation
- Bees discriminate between mutants with small intraspecific differences in visible color
- Major shift in pollinator preference by a single mutation of large phenotypic effect

Authors

Martina N. Lüthi, Andrea E. Berardi, Therese Mandel, Loreta B. Freitas, Cris Kuhlemeier

Correspondence

cris.kuhlemeier@ips.unibe.ch

In brief

UV color is a major determinant of pollinator preference. Lüthi et al. mutate *MYB-FL*, the key regulator of flavonol biosynthesis in *Petunia*. Hawkmoths prefer the UV-absorbent wild type, whereas bees prefer the mutant. This work represents a striking example of a single mutation of large phenotypic effect on pollinator-mediated reproductive isolation.

Lüthi et al., 2022, Current Biology *32*, 1–14
December 19, 2022
https://doi.org/10.1016/j.cub.2022.11.006

CellPress

Current Biology

CellPress

Article

Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference

Martina N. Lüthi,[1,3] Andrea E. Berardi,[1,4] Therese Mandel,[1] Loreta B. Freitas,[2] and Cris Kuhlemeier[1,5,*]
[1]Institute of Plant Sciences, University of Bern, Altenbergrain 21, 3013 Bern, Switzerland
[2]Department of Genetics, Universidade Federal do Rio Grande do Sul, POB 15053, Porto Alegre, 91501970 Rio Grande do Sul, Brazil
[3]Present address: Institute of Terrestrial Ecosystems, ETH Zurich, Universitätstrasse 16, 8092 Zurich, Switzerland
[4]Present address: Harvard University Herbaria, 22 Divinity Avenue, Cambridge, MA 02138, USA
[5]Lead contact
*Correspondence: cris.kuhlemeier@ips.unibe.ch
https://doi.org/10.1016/j.cub.2022.11.006

SUMMARY

Understanding the molecular basis of reproductive isolation and speciation is a key goal of evolutionary genetics. In the South American genus *Petunia*, the R2R3-MYB transcription factor *MYB-FL* regulates the biosynthesis of UV-absorbing flavonol pigments, a major determinant of pollinator preference. *MYB-FL* is highly expressed in the hawkmoth-pollinated *P. axillaris*, but independent losses of its activity in sister taxa *P. secreta* and *P. exserta* led to UV-reflective flowers and associated pollinator shifts in each lineage (bees and hummingbirds, respectively). We created a *myb-fl* CRISPR mutant in *P. axillaris* and studied the effect of this single gene on innate pollinator preference. The mutation strongly reduced the expression of the two key flavonol-related biosynthetic genes but only affected the expression of few other genes. The mutant flowers were UV reflective as expected but additionally contained low levels of visible anthocyanin pigments. Hawkmoths strongly preferred the wild-type *P. axillaris* over the *myb-fl* mutant, whereas both social and solitary bee preference depended on the level of visible color of the mutants. *MYB-FL*, with its specific expression pattern, small number of target genes, and key position at the nexus of flavonol and anthocyanin biosynthetic pathways, provides a striking example of evolution by single mutations of large phenotypic effect.

INTRODUCTION

Most plants are rooted in the soil and are prevented from actively seeking a mating partner in the way animals do. Male gametes, packed in resilient pollen, can be transferred by wind or water, but such abiotic vectors are rather inefficient.[1] Depending on the geographical region, 78%–94% of flowering plants are pollinated by animals,[2] demonstrating their importance for plant reproduction. In this mutually beneficial interaction, the pollinator transfers pollen between individual plants and is rewarded for its services with a high-energy food source, such as nectar. Floral color, scent, and morphology enhance the specificity of the interaction and further promote the preferential transfer of pollen to conspecific stigmas.[3] However, outsourcing pollen transfer to animal vectors carries the risk of failure when pollinators become unavailable or the local pollinator assemblage changes. Under such circumstances, adaptation to new pollinators can be beneficial as has happened frequently in many taxa, and it is widely accepted that animal-mediated pollination has promoted speciation and driven the rapid diversification of the angiosperms.[4]

A major theme in contemporary pollination research is the elucidation of the molecular-genetic basis of shifts in pollinator preference. Classical theory has long held that phenotypic change proceeds through many individual mutations, each of small phenotypic effect, as small mutations allow descendants to slowly diverge from the parental population without removing them far from their fitness optimum.[5] However, experimental data from genetic model organisms provided convincing evidence for mutations in single genes causing major phenotypic effects, an impressive early example being the establishment of apical dominance during the domestication of maize.[6] Over the past years, it has become clear that mutations of major phenotypic effect in single genes do contribute to adaptation, reproductive isolation, and speciation.[7–9] Moreover, it is difficult to reconcile gradual change with sympatric speciation, as ongoing gene flow and recombination will have homogenizing effects on populations.[10,11] The plausibility of mutations of major effect when selection is strong has a solid theoretical underpinning.[12,13] Loss of an established pollinator is likely to displace a plant far from its fitness optimum, a scenario that may favor mutations of large effect to help a pollinator adapt toward a new optimum. Indeed, there is substantial evidence that adaptation to a new pollinator involves loci of large phenotypic effect.[14–18]

Plants attract specific pollinators by combinations of floral traits such as color, scent, and morphology, so-called pollination syndromes. Pollinators can perceive colors not only in the visible range but also in the UV range.[19–22] While hawkmoths simply

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

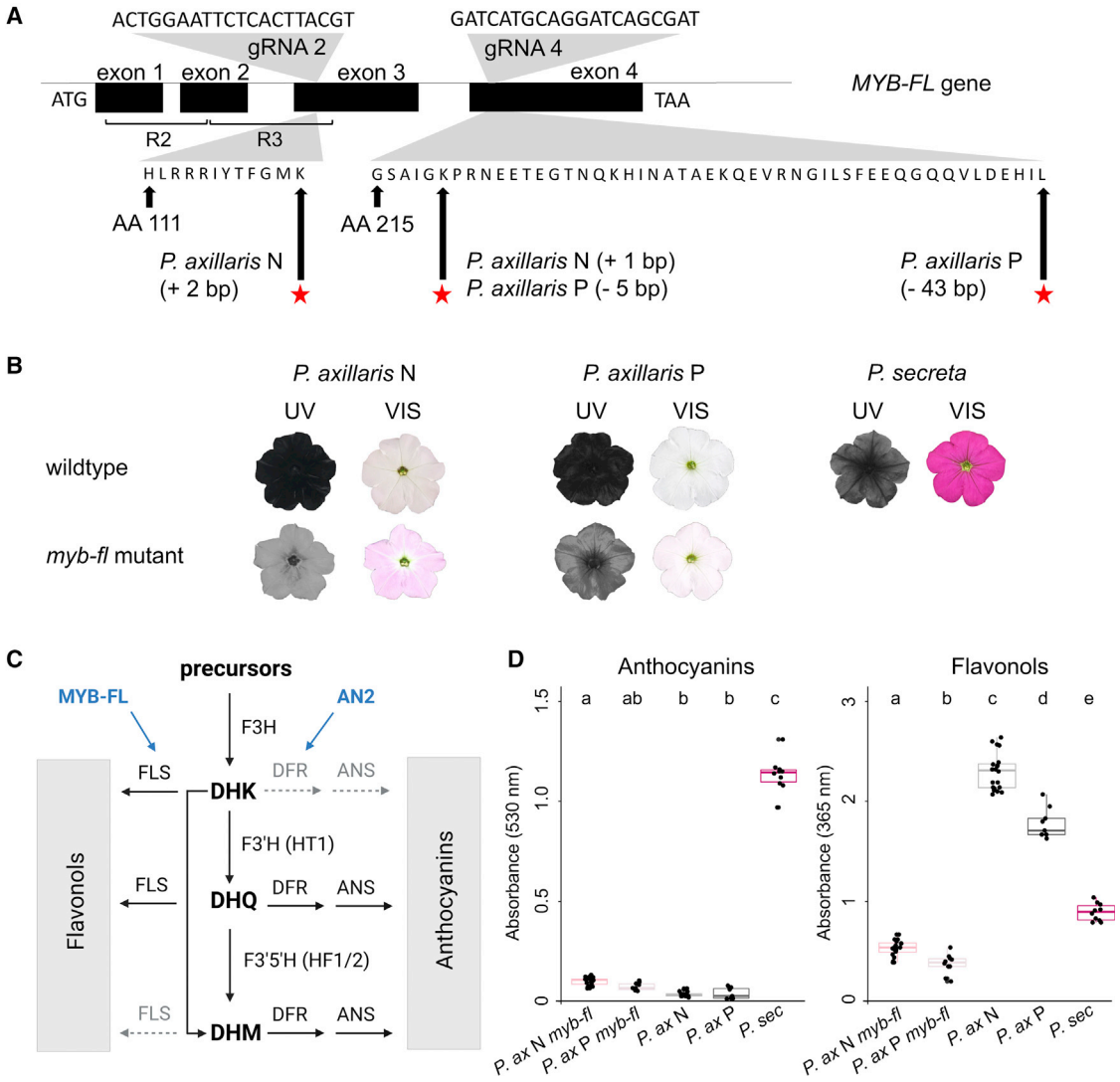


Figure 1. Targeting the *MYB-FL* gene via CRISPR-Cas9

(A) Two independent mutant lines of *P. axillaris* accessions N and P were generated, all leading to truncated proteins. Red asterisks indicate early stop codons.

(B) The *myb-fl* mutant flowers of both *P. axillaris* accessions are UV reflective. *P. axillaris* N mutants showed a clear pink color, while the *P. axillaris* P mutants were only faintly pink.

(C) Flavonols and anthocyanins are produced through individual branches of the flavonoid biosynthetic pathway. Enzymes needed for individual steps are depicted next to the respective arrow. Substrates are in bold. Transcription factors *AN2* and *MYB-FL* (highlighted in blue) activate DFR/anthocyanidin synthase (ANS) and FLS, respectively. *Petunia* DFR does not accept DHK as a substrate,[28] and FLS has low activity on DHM[29] (light gray color). Abbreviations: for genes that have different names in *Petunia*, we state the *Petunia* name in parentheses. DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin; F3H, flavonone 3-hydroxylase; F3′H (HT1), flavonoid-3′-hydroxylase, encoded by HT1; F3′5′H (HF1/2), flavonoid-3′5′-hydroxylase, encoded by HF1 and HF2; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase.

(D) Total flavonol and anthocyanin absorbance values of the wild-type and mutant lines as determined through spectrophotometric analysis. Letters indicate statistically significant groups calculated using a one-way ANOVA with Tukey's post hoc comparisons (full statistics in Table S3).

prefer UV-absorbing flowers, UV preference for bees is more complex.[18,23,24] In fact, for white-colored flowers, UV reflectance can significantly prolong a bee's search time,[25] whereas for red-colored flowers, bees prefer UV-reflecting flowers over UV-absorbing ones.[26] Both the visible and UV color displays of a flower are therefore vital in determining pollinator visitation. The major floral UV and visible color pigments responsible for floral color are synthesized by the flavonoid biosynthetic pathway. Two main branches of this pathway produce the visible color pigments: the anthocyanins and UV-absorbing flavonols (Figure 1C).[27] Anthocyanins can range in color from red to purple to blue, while flavonols are responsible for UV-absorbent flowers or UV patterns not visible to the human eye.

The South American genus *Petunia* (*Solanaceae*) offers a compelling system to study color transitions and associated pollinator shifts. The genus comprises species with distinct pollination syndromes, and the biochemistry and genetics of the flavonoid biosynthetic pathway have been extremely well

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

characterized.[30,31] Species in the long-tube clade display the most discrete phenotypic differences. *P. axillaris* displays white, UV-absorbing flowers emitting a rich blend of volatiles to attract nocturnal hawkmoths.[18,32,33] *P. exserta* has red flowers that are UV reflective, attracting hummingbirds as their primary pollinator.[34] *P. secreta* has magenta-colored, UV-reflecting flowers, primarily visited by solitary bees of the genus *Pseudagapostemon*.[35–37] Multiple major effect genes responsible for floral signal phenotypic differences that are associated with shifts in pollinator attraction have been identified.[14,17,18,38–40]

The combination of molecular-genetic data with phylogenetic analyses provides strong evidence that the common ancestor of the long-tube clade was likely hawkmoth pollinated with subsequent shifts to bees and hummingbirds.[39,40] *P. axillaris* and *P. secreta* in particular have similar floral morphologies, provide a nectar reward, and grow in sympatry. The regain of visible color in *P. secreta* was simple: a 2-bp deletion in the gene encoding the MYB transcription factor *AN2* restored the reading frame and anthocyanin biosynthesis.[39] Loss of UV color was achieved by the inactivation of *MYB-FL*.[18,39] The close phylogenetic relationship and the knowledge of causative genes make the *P. axillaris*/*P. secreta* species pair particularly interesting to study the effect of individual genetic changes on pollinator preference.

Introgression lines have been widely used to study the effect of single loci on pollinator behavior. A classic example is the behavioral assays in *Mimulus* where introgressions at the *YUP* flower color locus demonstrated differential pollinator preference.[41] However, even after generations of backcrossing, an introgressed region will include many linked genes with potentially confounding effects. This is particularly relevant in interspecific crosses, where recombination may be suppressed.[42] To rigorously test the effect of a single gene, behavioral assays must be performed comparing isogenic lines that only differ in the gene under study. Rare examples of such an approach are the transgenic overexpression of AN2 in *P. axillaris*[14] and the reduction of ocimene synthase expression in *Mimulus* by RNAi.[43] *Mixta* and *nivea* mutants in *Antirrhinum*, where single genes have been altered that affect flower color appearance, are rare examples where pollinator behavioral assays have been performed on single gene mutants, elegantly demonstrating their effects on bumblebee behavior.[44,45]

The *Petunia MYB-FL* gene offers an attractive system to test the precise impact of a single gene mutation on the flavonoid biosynthetic pathway, floral UV pigmentation phenotype, pollinator preference, and ultimately on reproductive isolation of species. To assess each of these aspects, we generated a knockout of the *MYB-FL* gene in two accessions of the hawkmoth-pollinated *P. axillaris* and tested the consequences of the mutation with respect to flavonoid biosynthesis, global gene expression, and innate pollinator preference. *MYB-FL* expression is highly restricted, and its knockout affects only a few genes in the genome. Moreover, we show that the loss of *MYB-FL* function has dramatic effects on the preference of both hawkmoths and bees.

RESULTS

CRISPR-Cas9-mediated knockout of *MYB-FL* gene causes a shift in the balance between UV and visible color

In order to inactivate the *MYB-FL* gene, we used a CRISPR-Cas9 approach with two gRNAs in *P. axillaris* accessions N and P (Figure 1A). Two independent mutations were generated in each background (Figure 1A). A 1- and 2-bp insertions were detected in the *P. axillaris* N background, while a 5- and 43-bp deletion were found in the *P. axillaris* P background. The mutations led to early stop codons through frameshifts, resulting in truncated *MYB-FL* proteins. All mutant lines showed a shift in floral UV phenotype; flowers were no longer UV absorbent but UV reflective (Figure 1B). Additionally, we observed a shift in the visible color phenotype, going from little to no anthocyanins (white) to shades of light pink. Interestingly, this shift in color differed between the *P. axillaris* accessions. In the *P. axillaris* N background, the corolla was light pink and easily observable by the human eye. By contrast, *P. axillaris* P mutants had a very faint pink corolla color (Figure 1B). Mutating *MYB-FL* in *P. axillaris* therefore not only induces a major reduction in UV absorption but also minor background-dependent increases in the visible range of the color spectrum.

To quantify the effects of the *MYB-FL* knockout on the flavonoid pigments that underlie visible color (anthocyanins) and UV color (flavonols) in *Petunia* (Figure 1C), we measured total flavonol and anthocyanin absorbance (relative concentration) in all lines using UV-Vis spectrophotometry. Total flavonol absorbance was significantly reduced in the *myb-fl* mutants compared with the wild-type lines, while anthocyanin absorbance was significantly higher in the *P. axillaris* N *myb-fl* mutants (Figure 1D; detailed statistics in Table S3). The differences in flavonol absorbance (and therefore their relative ratios) are marked between the mutant and wild-type lines, with much lower flavonol absorbance in the mutants than wild types. We did detect slightly higher anthocyanin absorbance in *P. axillaris* N *myb-fl* mutants compared with *P. axillaris* P *myb-fl* mutants, corresponding to the difference in the intensity of visible pink color (Figures 1B and 1D). Overall, *myb-fl* lines showed a reduction in flavonols responsible for UV color as well as an elevation in anthocyanin pigments responsible for visible color.

MYB-FL specifically affects floral UV-Vis absorption

To determine possible pleiotropic effects of the *MYB-FL* mutation, we measured several key traits that could potentially influence pollinator preference. Corolla size, pistil and stamen length, nectar volume and sugar concentration, and pollen germination rate showed no differences between wild-type and mutant lines (Figures 2A–2E). Epidermal cell shape has been reported to affect the efficiency of bee foraging[44,45] but is often overlooked. While the tested wild species (*P. secreta* and *P. axillaris* N and P) showed subtle differences in shapes of conical cells (Figure 2I), no differences were detected in cell shape between the *myb-fl* mutants and their corresponding wild-type lines. Similarly, an analysis of scent production revealed no differences between the *myb-fl* mutants and the respective wild-type lines for the major volatiles methylbenzoate, benzaldehyde, and isoeugenol/eugenol (Figures 2F–2H). In summary, the *myb-fl* mutation alters UV-Vis color without confounding effects on other major floral traits with potential relevance in pollinator preference.

MYB-FL is specifically expressed in the floral limb and has a limited number of target genes in the flavonoid pathway

Tissue specificity of *MYB-FL* expression was investigated by qRT-PCR in floral organs and leaves. Levels of *MYB-FL*

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

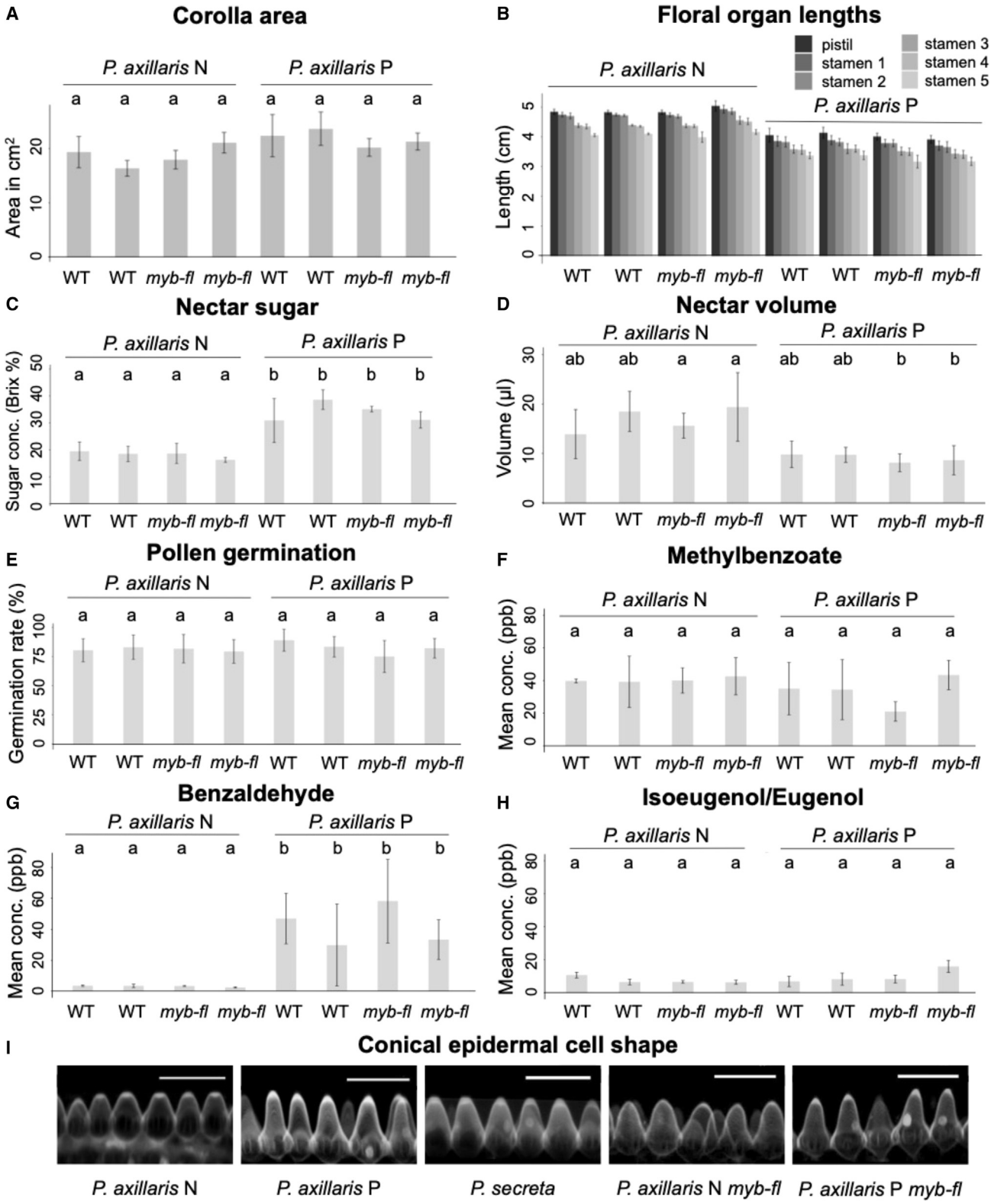


Figure 2. Phenotypic measurements of *myb-fl* mutant lines compared to wild-type *P. axillaris*

All bars depict mean values ± SD. Statistics were calculated using a one-way ANOVA with Tukey's post hoc comparisons (Table S3). Two independent mutant lines are shown for each *P. axillaris* line.

(A) Corolla areas for mutant and wild-type lines.

(legend continued on next page)

expression were negligible in all tested organ types except in floral limbs for both *P. axillaris* N and P (Figure 3A). In order to study changes in the expression of genes potentially regulated by *MYB-FL*, an RNA-seq experiment was conducted with two independent *myb-fl* mutants as well as the isogenic *P. axillaris* N. Given the observed changes in both UV and visible floral phenotypes, we investigated whether the mutation in *MYB-FL* affected the expression of downstream flavonoid biosynthetic genes as well as anthocyanin biosynthetic genes. The expression of *MYB-FL* itself is significantly reduced, possibly due to nonsense-mediated decay.[46] Only two genes, *FLS* and *HT1/F3'H*, are strongly downregulated in the mutant (Figure 3B; see Figure S1 for qRT-PCR data). FLS catalyzes the synthesis of flavonols from their dihydroflavonol precursors and is the only enzyme exclusively functioning in flavonol synthesis.[29] HT1/F3′H catalyzes the hydroxylation of the precursor dihydrokaempferol (DHK) into dihydroquercetin (DHQ), the immediate precursor of quercetin if modified by FLS and cyanidin if modified by dihydroflavonol-4-reductase (DFR).[29] Most of the anthocyanin biosynthetic genes were slightly higher expressed in the mutant, although in most cases not significantly so, and furthermore not confirmed by qRT-PCR (Figures 3B and S1). We therefore consider it likely that the gain in anthocyanins in the mutant is due to release from competition for their common substrates, the dihydroflavonols (Figure 1C). In summary, inactivation of *MYB-FL* caused a strong decrease in the expression of the two genes involved in flavonol synthesis (*FLS*, *HT1/F3′H*) and at most a marginal increase in the expression of anthocyanin biosynthetic genes. This points toward *MYB-FL* being specific in its targets.

Flavonols are important cues for pollinators, but they also fulfill other functions such as protection against high-light stress and inhibition of polar auxin transport.[47–50] To assess effects of the *myb-fl* mutation beyond the flavonoid biosynthetic pathway, global changes in gene expression between the *myb-fl* and wild-type lines were studied in petal limb tissue. Only 55 genes were significantly differentially expressed (DE), using a filter of a log2-fold change value greater than 1 or less than −1 and a p value < 0.05 (Table S2). Of these, 43 were downregulated and 12 were upregulated in the mutant. Several of the DE genes are potentially involved in high-light-induced stress responses (e.g., DNA photolyase Peaxi162Scf00034g01311 or cryptochrome/DNA photolyase Peaxi162Scf00213g01024; Table S2). *P. axillaris* typically grows in high-light environments,[51,52] and therefore flavonols might play a protective role in petals. We conclude that *MYB-FL* has a highly restricted expression pattern and affects only a small set of target genes.

Distinct innate pollinator preference of wild *Petunia* species

The evolution from hawkmoth to bee pollination in *Petunia* involved changes in a combination of floral traits, one of them being the loss of UV color.[18,39] The *myb-fl* mutants make it possible to disentangle the effect of changes in UV absorption from other floral traits on pollinator behavior. To assess innate pollinator preference, we set up pairwise comparisons between *P. axillaris* N and P and their isogenic *myb-fl* mutants. Three different pollinators were used (n = 20 for each comparison): the hawkmoth *Manduca sexta*, a pollinator of *P. axillaris* in the wild;[53] the bumblebee *B. terrestris*, a popular model organism; and the solitary bee *Osmia cornuta* (*O. cornuta*), which is closer in size and life cycle to the natural pollinator of *P. secreta* (solitary bee of the genus *Pseudagapostemon*) than to that of *B. terrestris*.[37] Choice experiments with *M. sexta*, a nocturnally active animal, were performed in a dark room supplemented with low UV light, similarly to UV reflectance from the moon during nighttime pollinator visitations. Experiments with *B. terrestris* and *O. cornuta* took place under natural light on sunny days (see STAR Methods for details). Animal behavior was recorded as first choice (the first plant visited) and total choices (all types of choices recorded during the 10-min duration of the experiments; bee choices are categorized as touch, visit, and forage, whereas hawkmoth choices are categorized as hover, probing, and forage; Figure S2).

To establish a baseline behavior, we first analyzed the preference of the three pollinators for the wild *Petunia* species. Hawkmoths significantly preferred *P. axillaris* for first and total choices, whereas bumblebees had a preference for *P. secreta* for first choice (significant only for total choices). This comparison evoked a more selective bee response from solitary bees than bumblebees: *O. cornuta* preferred *P. secreta* for both first and total choices and displayed very few visits to *P. axillaris* overall (Figure 4A; for separated data on types of choices, see Table S5). This demonstrates differences in innate preference between hawkmoths and bees as well as differences in the strength of the selectiveness between bee species.

Hawkmoths strongly prefer wild-type *P. axillaris* over isogenic *myb-fl* mutants

In the comparison between *P. axillaris* N and its isogenic *myb-fl* mutant, hawkmoths significantly preferred the wild-type flowers over the *myb-fl* mutant flowers for their first choice as well as the total number of choices (Figure 4B). The same was observed for hovering, probing, and foraging (Table S5). In the absence of UV light, the moths did not discriminate between wild type and mutant, clearly indicating that discrimination depends on the presence of UV light (Figure S3). Note that even under these conditions, the moths do not wander aimlessly around but efficiently approach the flowers, presumably guided by olfactory cues. Bees preferred the mutant over the wild type (not significant for *O. cornuta*, first choice). In the comparison between *P. axillaris* P and the isogenic *myb-fl*

(B) Pistil and stamen lengths. Each of the five stamens was measured individually.
(C and D) Sugar concentration (C) and volume of nectar (D).
(E) Pollen germination rates 2 h post inoculation in germination medium were determined.
(F–H) Scent volatiles methylbenzoate (F), benzaldehyde (G), and isoeugenol/eugenol (H) were analyzed for the mutant and wild-type lines via proton transfer reaction-mass spectrometry (PTR-MS).
(I) Conical epidermal cell shapes of three natural *Petunia* accessions as well as two *myb-fl* mutant lines. Images were acquired using confocal microscopy with propidium iodide-stained samples. Scale bars represent 50 μM in length.

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

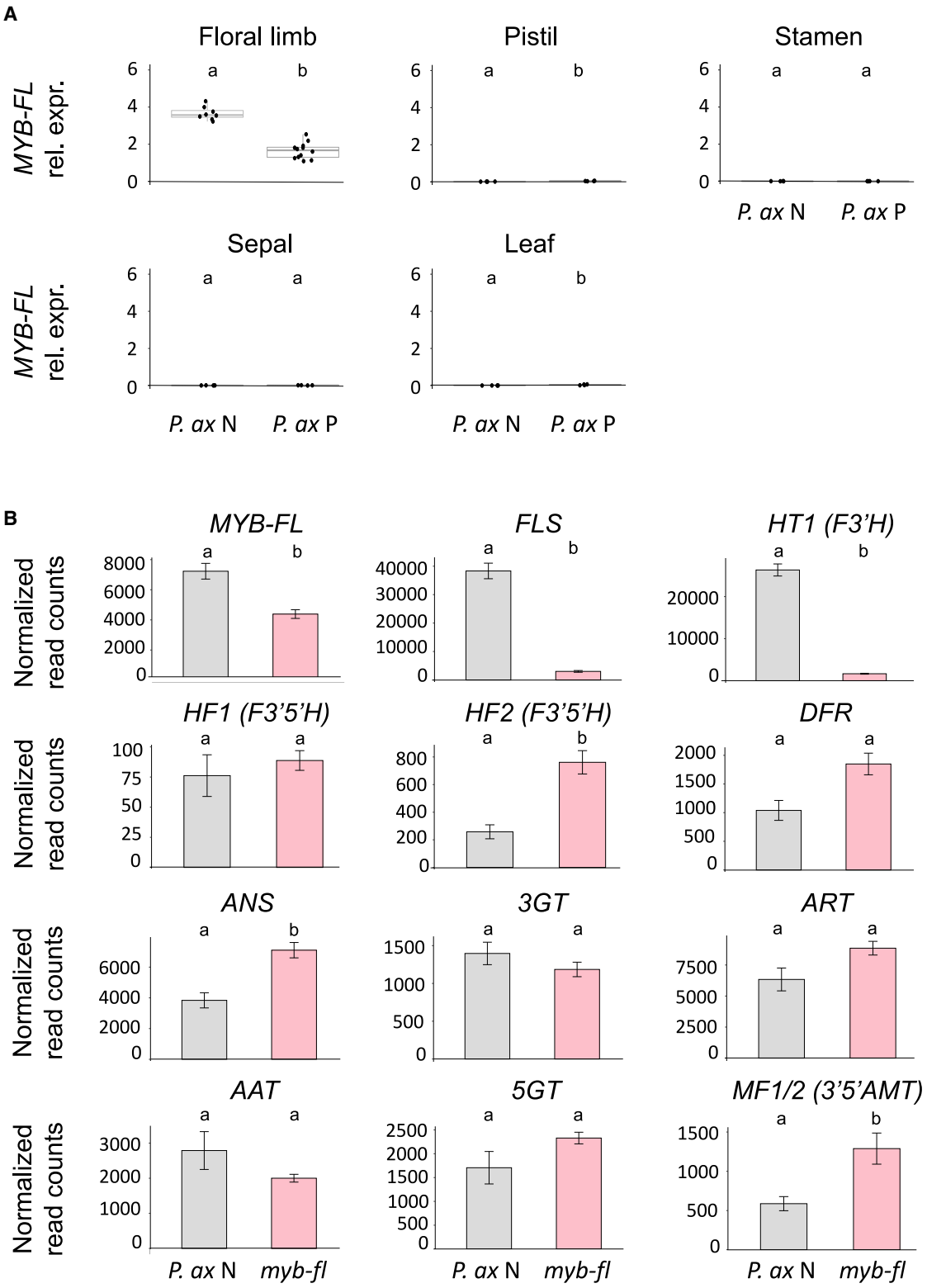


Figure 3. *MYB-FL* is specifically expressed in the floral limb and strongly induces expression of key genes involved in flavonol synthesis

(A) Organ specificity of *MYB-FL* expression was quantified in wild-type lines of both *P. axillaris* N and P through qRT-PCR. Expression is shown relative to the expression of the three housekeeping genes *SAND*, *RAN1*, and *ACTIN11*. Statistics were calculated using a Mann-Whitney U test; letters depict significant differences (full statistics in Table S4).

(legend continued on next page)

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

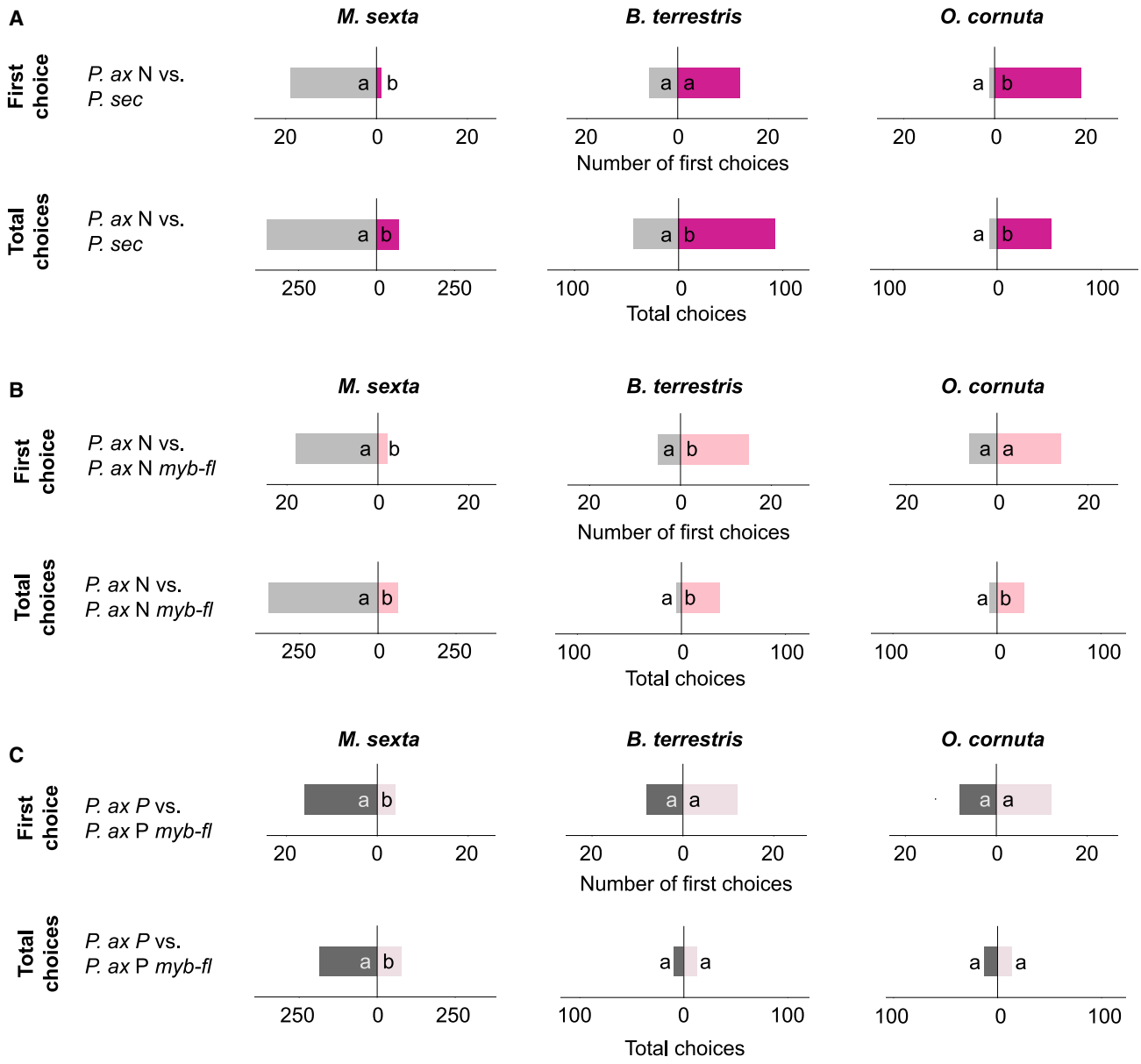


Figure 4. Hawkmoths strongly prefer wild-type *P. axillaris* over isogenic *myb-fl* mutants

Graphs show comparisons with three pollinator species: *M. sexta*, *B. terrestris*, and *O. cornuta* (n = 20 pollinators per comparison). Number of first choices as well as total number of choices are shown in each panel (see also Figure S3). Plant species labels on the left side of the graphs show the different pairwise comparisons (light gray, *P. axillaris* N; light pink, *P. axillaris* N *myb-fl*; dark gray, *P. axillaris* P; faint pink, *P. axillaris* P *myb-fl*). Statistical differences between groups were calculated using an exact binomial test and are depicted by letters (Table S5; see also Table S6).

(A) Comparison of wild-type lines *P. axillaris* N versus *P. secreta* for all three pollinator types.

(B) Comparison of *P. axillaris* N versus *P. axillaris* N *myb-fl* mutant for all three pollinator types.

(C) Comparison of *P. axillaris* P versus *P. axillaris* P *myb-fl* mutant for all three pollinator types.

mutant, hawkmoths strongly preferred the wild type (Figure 4C), almost identical to what was observed in Figure 4B. By contrast, both bumblebees and solitary bees displayed no significant preference for either genotype. Therefore, the loss of *MYB-FL* activity compromises the attraction of hawkmoths in both *P. axillaris* N and P, whereas bees have a preference

(B) Differential gene expression was measured in *P. axillaris* N *myb-fl* mutants and wild-type plants by RNA-seq. Read counts were normalized with rlog-transformation (full dataset and statistics in Tables S1 and S2; see also Figure S1). Bars show means of mapped reads for 8 biological replicates. Error bars represent ± SE. Letters depict significant differences ($p < 0.05$). 3GT, anthocyanin-3-glucosyltransferase; ART, anthocyanin rhamnosyltransferase; AAT, anthocyanin acyltransferase; 5GT, anthocyanin 5 glucosyltransferase; MF1/2 (3′5′AMT), 3′5′-anthocyanin methyltransferase encoded by *MF1* and *MF2*. For additional abbreviations, see Figure 1C.

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

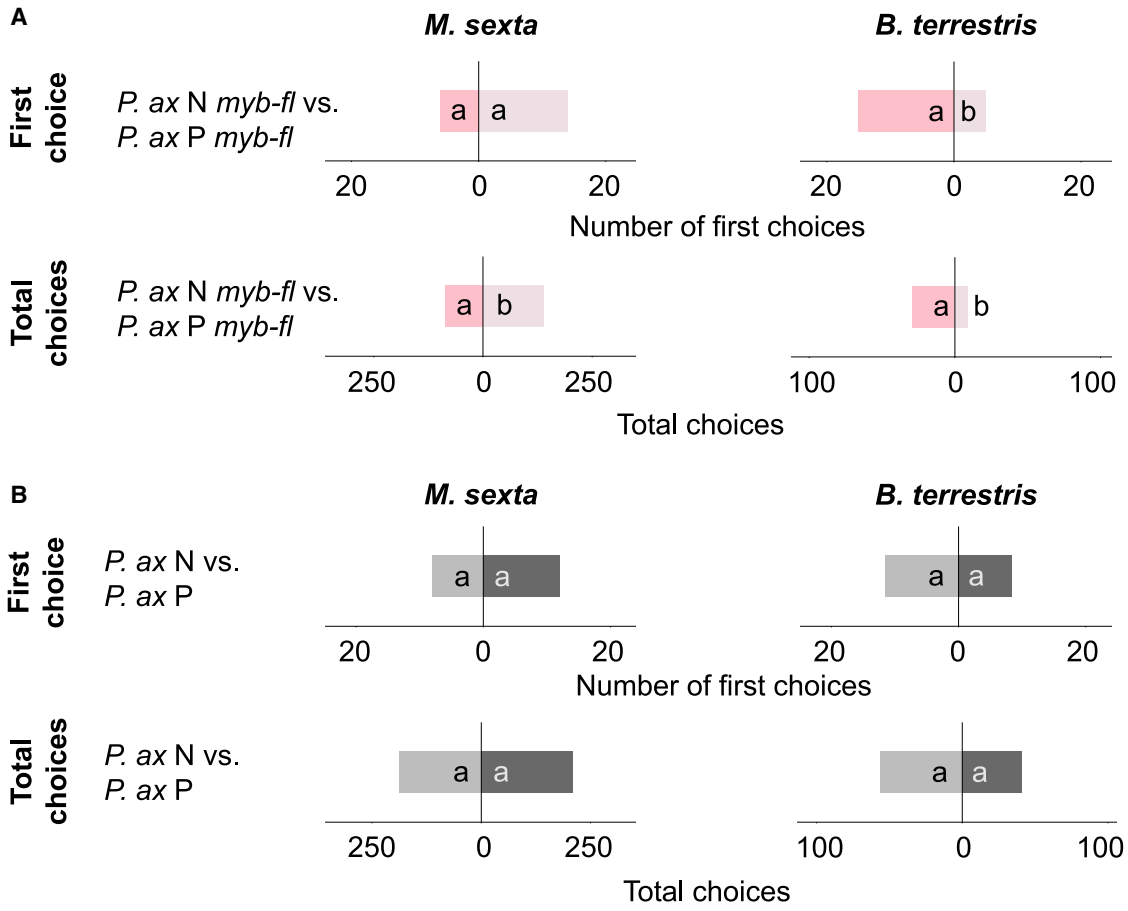


Figure 5. Intraspecific differences in visible color between *myb-fl* mutants affect pollinator choice

Pairwise comparisons were run for two pollinator species: *M. sexta* and *B. terrestris* (n = 20 pollinators per comparison). Bars display the number of first choices and total number of choices for each 10-min experiment (light gray, *P. axillaris* N; light pink, *P. axillaris* N *myb-fl*; dark gray, *P. axillaris* P; faint pink, *P. axillaris* P *myb-fl*). Plant species labels on the left side of the graphs show the different pairwise comparisons. Statistical differences between groups were calculated using an exact binomial test and are depicted by letters (Table S5; see also Table S6).

(A) Comparison of both *myb-fl* mutants, *P. axillaris* N *myb-fl* mutant versus *P. axillaris* P *myb-fl* mutant.

(B) Comparison of both *P. axillaris* wild types, *P. axillaris* N versus *P. axillaris* P.

for the mutant in the *P. axillaris* N background but not in the *P. axillaris* P background.

Intraspecific differences in visible color between *myb-fl* mutants affect bee preference

P. axillaris N and P are almost identical in floral traits relevant for pollinator behavior (Figure 2), but an obvious difference between their *myb-fl* mutants is the intensity of the visible color: light pink, easily visible by the human eye, in *myb-fl* mutants of *P. axillaris* N and barely visible in *P. axillaris* P mutants (Figure 1B). To analyze the effect of these differences in visible color, we tested pollinator behavior with an array containing the two mutants from which to choose (Figure 5A). In control experiments, hawkmoths and bees equally visited each of the wild-type lines (Figure 5B). In the mutant comparisons, hawkmoths displayed a weak preference for the pale *P. axillaris* P mutant, whereas bumblebees strongly preferred the darker colored *P. axillaris* N mutant for both first choice and total choices. We conclude that the loss of *MYB-FL* activity causes a stronger shift toward bee pollination in *P. axillaris* N due to its stronger visible color.

Bees strongly prefer *P. secreta* over the *myb-fl* mutants

If the *myb-fl* mutants represent an intermediate stage of adaptation toward a different pollinator group, they should be less attractive to a hawkmoth pollinator and more attractive to a bee pollinator. Therefore, we compared the *myb-fl* mutants of either *P. axillaris* N or P backgrounds with *P. secreta* (Figure 6). In these comparisons, all flowers are UV reflective, but *P. secreta* flowers have a stronger magenta anthocyanin pigmentation than the mutants. Hawkmoths preferred the *P. axillaris* N *myb-fl* mutants over *P. secreta* (Figure 6A; not significant for first choice for *P. axillaris* N *myb-fl*). In this same comparison, bumblebees preferred *P. secreta* (not significant for total choices), and solitary bees preferred *P. secreta* as well (not significant for first choice). Therefore, in the absence of UV-absorbing flavonols, hawkmoths have a distinct tendency to visit the *P. axillaris* N mutant and bees to visit *P. secreta*. In the case of the *P. axillaris*

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

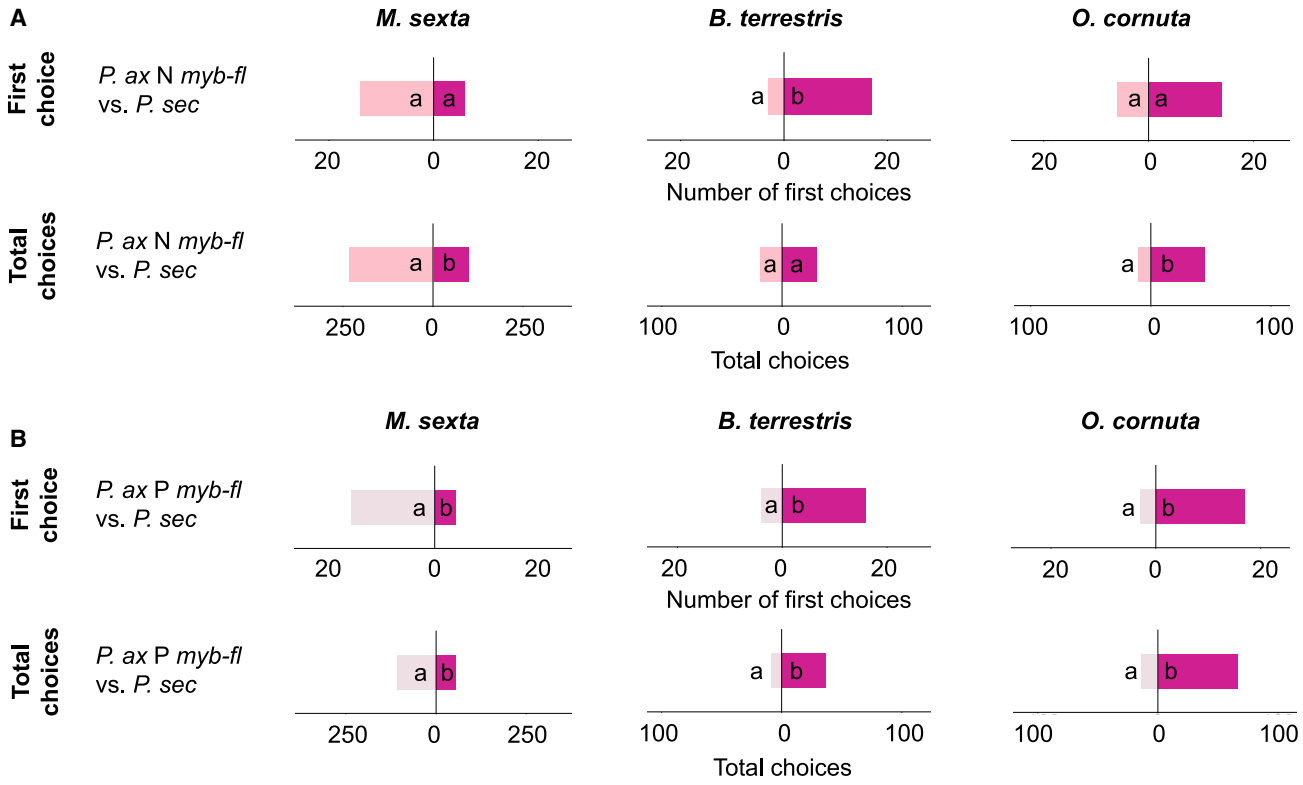


Figure 6. Bees strongly prefer *P. secreta* over *myb-fl* mutants

Graphs show comparisons of *myb-fl* mutants with *P. secreta*, using three pollinator species: *M. sexta*, *B. terrestris*, and *O. cornuta* (n = 20 pollinators per comparison). Number of first choices as well as total number of choices are shown in each panel. Plant species labels on the left side of the graphs show the different pairwise comparisons (magenta, *P. secreta*; light pink, *P. axillaris* N *myb-fl*; faint pink, *P. axillaris* P *myb-fl*). Statistical differences between groups were calculated using an exact binomial test and are depicted by letters (Table S5; see also Table S6).

P mutant, the same preferences were observed but were statistically significant in all comparisons: hawkmoths preferred the mutant over *P. secreta*, whereas bees preferred *P. secreta* (Figure 6B). The clearer preferences in the comparisons with the *P. axillaris* P mutants compared with the assays with *P. axillaris* N mutants are most likely due to the differences in visible color observed between the mutants. In summary, the loss of *MYB-FL* activity seems to be enough to completely shift hawkmoth preference away from the mutants when comparing them with the isogenic *P. axillaris* wild types, but it does not suffice to make the mutants equivalent to *P. secreta* in the eyes of a hawkmoth pollinator. This supports the hypothesis that the *P. axillaris myb-fl* mutants represent intermediate stages, are no longer adapted to their original pollinator, but are not yet completely adapted to a novel pollinator.

DISCUSSION

Shifts in pollinators are widely recognized as a driving force of floral diversification and speciation in the angiosperms.[4,54,55] Testing the evolutionary role and specific effects of single gene mutations in potential speciation genes remains challenging in many species, and there are few instances in the literature identifying single mutations while also demonstrating a direct influence of the mutation on pollinator behavior.[14,44,45] We constructed a CRISPR mutation in the floral UV-controlling transcription factor *MYB-FL* that mimics the loss-of-function alleles found in nature[18,39] and studied its specific impact on pollinator preference and thereby on reproductive isolation and speciation in *Petunia*.

MYB-FL expression in *P. axillaris* is tightly restricted in time and developmental space, and it has only a few target genes (Figure 3A; Table S2).[18] Such properties may well be a prerequisite for genes of large phenotypic effect to induce a favorable evolutionary change with a minimum of undesirable side effects. Specific expression patterns of R2R3-MYBs have also been described in other plants—for flavonol synthesis as well as other traits relevant to pollination.[56–61] It has even been suggested that pollination syndromes are preferably controlled by MYB factors, reminiscent of the prominent role of MADS-box factors in the determination of floral organ whorl identity.[62] A classic example from the non-plant literature is the role of *Hox* genes in *Drosophila* segmentation,[63] illustrating the idea that complex traits could have evolved through the diversification and specialization of particular classes of transcription factors. Flavonoid synthesis is a beautiful example of a conserved pathway with variations that adapt it to the needs of individual species, moth pollination in *Petunia*, or for example, the exquisite color patterning that guides bumblebees in *Anthirrhinum majus*.[64]

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

Major shifts in pollinator preference due to a single gene with dual phenotypic effects

The pollinator choice assays between isogenic genotypes demonstrate that mutation of *MYB-FL* has a major impact on reproductive isolation in *Petunia*. The primary pollinator of *P. axillaris* (hawkmoth) significantly preferred the wild-type plants over the *myb-fl* mutants (Figures 4B and 4C), supporting the suggested innate preference for UV-absorbing *Petunia* flowers.[18] Although inactivation of *MYB-FL* shifts pollination away from hawkmoths, the mutation alone is not enough to fully shift to bee pollination; secondary pollinators of *P. axillaris* (bumblebees and solitary bees) were still able to discriminate between the *myb-fl* mutant and *P. secreta* flowers, especially in the *P. axillaris* P background (Figures 6A and 6B). This retained discrimination by bee species between these UV-reflective choices (but different in visible anthocyanin pigments) suggests that reproductive isolation still occurs between the *myb-fl* mutant and *P. secreta*.

Additionally, our phenotypic analyses showed a dual phenotypic effect due to mutation to *MYB-FL* with a strong reduction in flavonol absorbance as well as a simultaneous increase in anthocyanin absorbance in the *myb-fl* mutants, compared with wild-type plants (Figure 1D). The UV-absorbing flowers were important cues for nighttime hawkmoth pollinators but did not have as strong of an effect on bee pollinators (Figure 4). *MYB-FL* activity is therefore not a prerequisite for daytime pollination to occur, and its loss of function is likely to free up dihydroflavonol precursors for anthocyanin production. Thus, this single mutation results in the loss of flavonols and gain of anthocyanins; both phenotypic traits that are favorable to shift pollinator preference from hawkmoth to bees. Such combined traits have the potential to increase a plant's reproductive success.[65] We also note that the innate preferences detected here could be easily transformed by insect learning in an ecological context in which these mutants arise (and are likely in very low frequency).

With its intermediate phenotype, the *myb-fl* mutant represents a transitional form of the evolutionary process that occurred during the shift from hawkmoth to bee pollination syndrome. Major phenotypic leaps were long thought to be deleterious, but theory now accepts the possibility of genes of large phenotypic effect,[66] and this case demonstrates experimentally that a leap large enough to attract a new pollinator can allow reproductive isolation to occur through changes in a low number of genes of large effect. However, in nature, *Petunia* visitation by a sole pollinator type is not always the case, with several documented secondary visitors.[14,37] For reproductive isolation to be maintained, a strong but not perfect pollinator preference may therefore suffice, especially if hybrids fail.[16,67,68]

UV color as an initial cue for hawkmoth preference

Comparing the two *myb-fl* mutants with different wild types allowed us to study the effect of UV-vis color on pollinator preference in more detail. In all the experimental setups where the plant lines differed in UV color (absorbent versus reflective), hawkmoths strongly preferred UV-absorbent flowers for first choice, regardless of the intensity of visible color, whereas preferences for UV-reflective flowers as a first choice were not equally strong for bee pollinators (Figure 4). Based on these findings, UV color seems to act as an initial and important cue during a hawkmoth's decision-making process to approach a flower but not during a bee's. While UV-absorbent flowers are attractive to hawkmoth pollinators,[18,23,69,70] flowers pollinated by diurnal pollinators often display UV patterning, such as nectar guides, that affects pollinator attraction.[71–74] The binary UV phenotype (absorbent/reflective) we used in our experiments may therefore be a less informative cue to bees than hawkmoths when deciding to approach a flower.

In our experiments, bees also seemed to rely more strongly on differences in the visible spectrum than hawkmoths. When presented with the two *myb-fl* mutants, both UV reflective but differing in intensity of visible color (Figure 5A), bumblebees significantly preferred the more visibly colored *P. axillaris* N mutant, whereas hawkmoths showed no significant preference for either mutant for first choice. This points toward a strong reliance on visible color during a bee's decision-making process, as has been demonstrated for diurnal pollinators.[75–79] Although the difference in visible color between the two mutants is rather subtle and not as strong as between wild-type *P. axillaris* and *P. secreta* (Figures 1B and 1D), it is detectable for bumblebees (Figure 5). When comparing these findings with the comparisons with *P. secreta*, bees clearly prefer *P. secreta* over the *P. axillaris* P *myb-fl* mutant (Figure 6B), but the preference is not as clear for the *P. axillaris* N *myb-fl* mutant (Figure 6A). In the absence of UV-absorbing flowers, even a minor addition of visible color already has a remarkably strong effect on bee preference (Figure 5A).

Reproductive isolation through multiple major effect genes

If the synthesis of high levels of flavonols limits anthocyanin production due to metabolic competition for common precursors, a decrease in flavonols is required before anthocyanins can be produced. This implies that the loss of *MYB-FL* activity is most likely an early mutational event in the evolution from hawkmoth to bee pollination. Through its dual phenotypic effect, mutation of *MYB-FL* may have made a strong initial contribution toward reproductive isolation during the divergence of the two visibly colored species (*P. secreta* and *P. exserta*) from the common ancestor.

Pollinators can integrate multiple cues to choose between plant species. Just like color, scent differences between species also affect pollinator preference.[43,70,80,81] Previous analyses of hawkmoth preferences for *Petunia* scent volatiles discovered that hawkmoths preferred scented to non-scented plants, but when presented with conflicting cues (scent and color), both cues were equivalent.[80] This demonstrates that a combination of cues (generally referred to as pollination syndromes) is necessary to make efficient choices. Additional genes of major phenotypic effect(s) have been described in *Petunia* and other systems, and it will be interesting to study their effects on pollinator preference, separately and in combination.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

- Lead contact
- Materials availability
- Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Color and UV image scoring
  - Measurement of additional floral traits
  - Pollen germination measurements
  - Quantification of flavonols and anthocyanins
  - Confocal microscopy of epidermal cells
  - Generating CRISPR constructs
  - Stable transformation of *P. axillaris*
  - DNA extractions and genotyping
  - RNA extractions, cDNA preparation and RT-qPCR
  - RNA sequencing
  - Read processing and differential expression
  - Behavioral experiments
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.11.006.

ACKNOWLEDGMENTS

This work was supported by grants from the Swiss National Science Foundation (31003A_159493 and 31003A_182340) and the European Union (ERC AdG 741354-RESPEC). We thank Lea Jäggi and Fabrizio Strasser for technical support, Marta Binaghi and Gina Cannarozzi for bioinformatics support, and Diane Bonnet for scientific illustrations. The HPC cluster of the University of Bern was used for computationally demanding processes (http://www.id.unibe.ch/hpc).

AUTHOR CONTRIBUTIONS

M.N.L., A.E.B., and T.M. performed laboratory work, and M.N.L. and A.E.B. executed data analyses. L.B.F. and C.K. supervised the project. M.N.L., A.E.B., and C.K. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: July 28, 2022
Revised: September 16, 2022
Accepted: November 3, 2022
Published: December 5, 2022

REFERENCES

1. Scopece, G., Cozzolino, S., Johnson, S.D., and Schiestl, F.P. (2010). Pollination efficiency and the evolution of specialized deceptive pollination systems. Am. Nat. *175*, 98–105. https://doi.org/10.1086/648555.
2. Ollerton, J., Winfree, R., and Tarrant, S. (2011). How many flowering plants are pollinated by animals? Oikos *120*, 321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x.
3. Armbruster, W.S. (2017). The specialization continuum in pollination systems: diversity of concepts and implications for ecology, evolution and conservation. Funct. Ecol. *31*, 88–100. https://doi.org/10.1111/1365-2435.12783.
4. Van der Niet, T., Peakall, R., and Johnson, S.D. (2014). Pollinator-driven ecological speciation in plants: new evidence and future perspectives. Ann. Bot. *113*, 199–211. https://doi.org/10.1093/aob/mct290.
5. Fisher, R.A. (1930). The Genetical Theory of Natural Selection (Clarendon Press). https://doi.org/10.5962/bhl.title.27468.
6. Doebley, J., Stec, A., and Hubbard, L. (1997). The evolution of apical dominance in maize. Nature *386*, 485–488. https://doi.org/10.1038/386485a0.
7. Nadeau, N.J., Pardo-Diaz, C., Whibley, A., Supple, M.A., Saenko, S.V., Wallbank, R.W.R., Wu, G.C., Maroja, L., Ferguson, L., Hanly, J.J., et al. (2016). The gene cortex controls mimicry and crypsis in butterflies and moths. Nature *534*, 106–110. https://doi.org/10.1038/nature17961.
8. Barrett, R.D.H., Laurent, S., Mallarino, R., Pfeifer, S.P., Xu, C.C.Y., Foll, M., Wakamatsu, K., Duke-Cohan, J.S., Jensen, J.D., and Hoekstra, H.E. (2019). Linking a mutation to survival in wild mice. Science *363*, 499–504. https://doi.org/10.1126/science.aav3824.
9. Todesco, M., Owens, G.L., Bercovich, N., Légaré, J.-S., Soudi, S., Burge, D.O., Huang, K., Ostevik, K.L., Drummond, E.B.M., Imerovski, I., et al. (2020). Massive haplotypes underlie ecotypic differentiation in sunflowers. Nature *584*, 602–607. https://doi.org/10.1038/s41586-020-2467-6.
10. Bolnick, D.I., and Fitzpatrick, B.M. (2007). Sympatric speciation: models and empirical evidence. Annu. Rev. Ecol. Evol. Syst. *38*, 459–487. https://doi.org/10.1146/annurev.ecolsys.38.091206.095804.
11. Stankowski, S., and Ravinet, M. (2021). Defining the speciation continuum. Evolution *75*, 1256–1273. https://doi.org/10.1111/evo.14215.
12. Orr, H.A. (1998). The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. Evolution *52*, 935–949. https://doi.org/10.1111/j.1558-5646.1998.tb01823.x.
13. Orr, H.A., and Coyne, J.A. (1992). The genetics of adaptation: a reassessment. Am. Nat. *140*, 725–742. https://doi.org/10.1086/285437.
14. Hoballah, M.E., Gübitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., Dell'Olivo, A., Arnold, M., and Kuhlemeier, C. (2007). Single gene–mediated shift in pollinator attraction in Petunia. Plant Cell *19*, 779–790. https://doi.org/10.1105/tpc.106.048694.
15. Rieseberg, L.H., and Blackman, B.K. (2010). Speciation genes in plants. Ann. Bot. *106*, 439–455. https://doi.org/10.1093/aob/mcq126.
16. Hopkins, R., and Rausher, M.D. (2012). Pollinator-mediated selection on flower color allele drives reinforcement. Science *335*, 1090–1092. https://doi.org/10.1126/science.1215198.
17. Amrad, A., Moser, M., Mandel, T., de Vries, M., Schuurink, R.C., Freitas, L., and Kuhlemeier, C. (2016). Gain and loss of floral scent production through changes in structural genes during pollinator-mediated speciation. Curr. Biol. *26*, 3303–3312. https://doi.org/10.1016/j.cub.2016.10.023.
18. Sheehan, H., Moser, M., Klahre, U., Esfeld, K., Dell'Olivo, A., Mandel, T., Metzger, S., Vandenbussche, M., Freitas, L., and Kuhlemeier, C. (2016). MYB-FL controls gain and loss of floral UV absorbance, a key trait affecting pollinator preference and reproductive isolation. Nat. Genet. *48*, 159–166. https://doi.org/10.1038/ng.3462.
19. Chen, D.M., Collins, J.S., and Goldsmith, T.H. (1984). The ultraviolet receptor of bird retinas. Science *225*, 337–340. https://doi.org/10.1126/science.6740315.
20. Kelber, A., Balkenius, A., and Warrant, E.J. (2003). Colour vision in diurnal and nocturnal hawkmoths. Integr. Comp. Biol. *43*, 571–579. https://doi.org/10.1093/icb/43.4.571.
21. Dyer, A.G., Paulk, A.C., and Reser, D.H. (2011). Colour processing in complex environments: insights from the visual system of bees. Proc. Biol. Sci. *278*, 952–959. https://doi.org/10.1098/rspb.2010.2412.
22. Stoddard, M.C., Eyster, H.N., Hogan, B.G., Morris, D.H., Soucy, E.R., and Inouye, D.W. (2020). Wild hummingbirds discriminate nonspectral colors. Proc. Natl. Acad. Sci. USA *117*, 15112–15122. https://doi.org/10.1073/pnas.1919377117.
23. White, R.H., Stevenson, R.D., Bennett, R.R., Cutler, D.E., and Haber, W.A. (1994). Wavelength discrimination and the role of ultraviolet vision in the feeding behavior of hawkmoths. Biotropica *26*, 427. https://doi.org/10.2307/2389237.
24. Kevan, P.G., Chittka, L., and Dyer, A.G. (2001). Limits to the salience of ultraviolet: lessons from colour vision in bees and birds. J. Exp. Biol. *204*, 2571–2580. https://doi.org/10.1242/jeb.204.14.2571.

25. Spaethe, J., Tautz, J., and Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. Proc. Natl. Acad. Sci. USA *98*, 3898–3903. https://doi.org/10.1073/pnas.071053098.

26. Chen, Z., Liu, C.-Q., Sun, H., and Niu, Y. (2020). The ultraviolet colour component enhances the attractiveness of red flowers of a bee-pollinated plant. J. Plant Ecol. *13*, 354–360. https://doi.org/10.1093/jpe/rtaa023.

27. Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. *126*, 485–493. https://doi.org/10.1104/pp.126.2.485.

28. Johnson, E.T., Ryu, S., Yi, H., Shin, B., Cheong, H., and Choi, G. (2001). Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. Plant J. *25*, 325–333. https://doi.org/10.1046/j.1365-313x.2001.00962.x.

29. Holton, T.A., Brugliera, F., and Tanaka, Y. (1993). Cloning and expression of flavonol synthase from Petunia hybrida. Plant J. *4*, 1003–1010. https://doi.org/10.1046/j.1365-313X.1993.04061003.x.

30. Tornielli, G., Koes, R., and Quattrocchio, F. (2009). The genetics of flower color. In Petunia, T. Gerats, and J. Strommer, eds. (Springer), pp. 269–299. https://doi.org/10.1007/978-0-387-84796-2_13.

31. Sheehan, H., Hermann, K., and Kuhlemeier, C. (2012). Color and scent: how single genes influence pollinator attraction. Cold Spring Harbor Symp. Quant. Biol. *77*, 117–133. https://doi.org/10.1101/sqb.2013.77.014712.

32. Hoballah, M.E., Stuurman, J., Turlings, T.C.J., Guerin, P.M., Connétable, S., and Kuhlemeier, C. (2005). The composition and timing of flower odour emission by wild Petunia axillaris coincide with the antennal perception and nocturnal activity of the pollinator Manduca sexta. Planta *222*, 141–150. https://doi.org/10.1007/s00425-005-1506-8.

33. Turchetto, C., Segatto, A.L.A., Beduschi, J., Bonatto, S.L., and Freitas, L.B. (2015). Genetic differentiation and hybrid identification using microsatellite markers in closely related wild species. AoB Plants *7*, plv084. https://doi.org/10.1093/aobpla/plv084.

34. Lorenz-Lemke, A.P., Mäder, G., Muschner, V.C., Stehmann, J.R., Bonatto, S.L., Salzano, F.M., and Freitas, L.B. (2006). Diversity and natural hybridization in a highly endemic species of Petunia (Solanaceae): a molecular and ecological analysis. Mol. Ecol. *15*, 4487–4497. https://doi.org/10.1111/j.1365-294X.2006.03100.x.

35. Stehmann, J.R., and Semir, J. (2005). New species of Calibrachoa and Petunia (Solanaceae) from Subtropical South America. Monogr. Syst. Bot. Mo. Bot. Gard *104*, 341–348.

36. Rodrigues, D.M., Turchetto, C., Callegari-Jacques, S.M., and Freitas, L.B. (2018). Can the reproductive system of a rare and narrowly endemic plant species explain its high genetic diversity? Acta Bot. Bras. *32*, 180–187. https://doi.org/10.1590/0102-33062017abb0296.

37. Rodrigues, D.M., Caballero-Villalobos, L., Turchetto, C., Assis Jacques, R., Kuhlemeier, C., and Freitas, L.B. (2018). Do we truly understand pollination syndromes in Petunia as much as we suppose? AoB Plants *10*, ply057. https://doi.org/10.1093/aobpla/ply057.

38. Quattrocchio, F., Wing, J., and Koes, R. (1999). Molecular analysis of the anthocyanin2 gene of Petunia and its role in the evolution of flower color. Pant Cell *11*, 1433–1444.

39. Esfeld, K., Berardi, A.E., Moser, M., Bossolini, E., Freitas, L., and Kuhlemeier, C. (2018). Pseudogenization and resurrection of a speciation gene. Curr. Biol. *28*, 3776–3786.e7. https://doi.org/10.1016/j.cub.2018.10.019.

40. Berardi, A.E., Esfeld, K., Jäggi, L., Mandel, T., Cannarozzi, G.M., and Kuhlemeier, C. (2021). Complex evolution of novel red floral color in Petunia. Plant Cell *33*, 2273–2295. https://doi.org/10.1093/plcell/koab114.

41. Bradshaw, H.D., and Schemske, D.W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. Nature *426*, 176–178. https://doi.org/10.1038/nature02106.

42. Hermann, K., Klahre, U., Moser, M., Sheehan, H., Mandel, T., and Kuhlemeier, C. (2013). Tight genetic linkage of prezygotic barrier loci creates a multifunctional speciation island in Petunia. Curr. Biol. *23*, 873–877. https://doi.org/10.1016/j.cub.2013.03.069.

43. Byers, K.J.R.P., Vela, J.P., Peng, F., Riffell, J.A., and Bradshaw, H.D. (2014). Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (Mimulus). Plant J. *80*, 1031–1042. https://doi.org/10.1111/tpj.12702.

44. Dyer, A.G., Whitney, H.M., Arnold, S.E.J., Glover, B.J., and Chittka, L. (2007). Mutations perturbing petal cell shape and anthocyanin synthesis influence bumblebee perception of Antirrhinum majus flower colour. Arthropod Plant Interact. *1*, 45–55. https://doi.org/10.1007/s11829-007-9002-7.

45. Whitney, H.M., Chittka, L., Bruce, T.J.A., and Glover, B.J. (2009). Conical epidermal cells allow bees to grip flowers and increase foraging efficiency. Curr. Biol. *19*, 948–953. https://doi.org/10.1016/j.cub.2009.04.051.

46. Nickless, A., Bailis, J.M., and You, Z. (2017). Control of gene expression through the nonsense-mediated RNA decay pathway. Cell Biosci. *7*, 26. https://doi.org/10.1186/s13578-017-0153-7.

47. Tattini, M., Guidi, L., Morassi-Bonzi, L., Pinelli, P., Remorini, D., Degl'Innocenti, E., Giordano, C., Massai, R., and Agati, G. (2005). On the role of flavonoids in the integrated mechanisms of response of Ligustrum vulgare and Phillyrea latifolia to high solar radiation. New Phytol. *167*, 457–470. https://doi.org/10.1111/j.1469-8137.2005.01442.x.

48. Agati, G., and Tattini, M. (2010). Multiple functional roles of flavonoids in photoprotection. New Phytol. *186*, 786–793.

49. Liu, Y., Fang, S., Yang, W., Shang, X., and Fu, X. (2018). Light quality affects flavonoid production and related gene expression in Cyclocarya Paliurus. J. Photochem. Photobiol. B *179*, 66–73. https://doi.org/10.1016/j.jphotobiol.2018.01.002.

50. Teale, W.D., Pasternak, T., Dal Bosco, C., Dovzhenko, A., Kratzat, K., Bildl, W., Schwörer, M., Falk, T., Ruperti, B., Schaefer, J.V., et al. (2021). Flavonol-mediated stabilization of PIN efflux complexes regulates polar auxin transport. EMBO J. *40*, e104416. https://doi.org/10.15252/embj.2020104416.

51. Turchetto, C., Fagundes, N.J.R., Segatto, A.L.A., Kuhlemeier, C., Solís Neffa, V.G., Speranza, P.R., Bonatto, S.L., and Freitas, L.B. (2014). Diversification in the South American Pampas: the genetic and morphological variation of the widespread *Petunia axillaris* complex (Solanaceae). Mol. Ecol. *23*, 374–389. https://doi.org/10.1111/mec.12632.

52. Turchetto, C., Segatto, A.L.A., Silva-Arias, G.A., Beduschi, J., Kuhlemeier, C., Bonatto, S.L., and Freitas, L.B. (2019). Contact zones and their consequences: hybridization between two ecologically isolated wild Petunia species. Bot. J. Linn. Soc. *190*, 421–435. https://doi.org/10.1093/botlinnean/boz022.

53. Venail, J., Dell'Olivo, A., and Kuhlemeier, C. (2010). Speciation genes in the genus Petunia. Philos. Trans. R. Soc. Lond. B Biol. Sci. *365*, 461–468. https://doi.org/10.1098/rstb.2009.0242.

54. Sapir, Y., and Armbruster, W.S. (2010). Pollinator-mediated selection and floral evolution: from pollination ecology to macroevolution. New Phytol. *188*, 303–306. https://doi.org/10.1111/j.1469-8137.2010.03467.x.

55. Schiestl, F.P., and Johnson, S.D. (2013). Pollinator-mediated evolution of floral signals. Trends Ecol. Evol. *28*, 307–315. https://doi.org/10.1016/j.tree.2013.01.019.

56. Mehrtens, F., Kranz, H., Bednarek, P., and Weisshaar, B. (2005). The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol. *138*, 1083–1096. https://doi.org/10.1104/pp.104.058032.

57. Verdonk, J.C., Haring, M.A., van Tunen, A.J., and Schuurink, R.C. (2005). ODORANT1 regulates fragrance biosynthesis in petunia flowers. Plant Cell *17*, 1612–1624. https://doi.org/10.1105/tpc.104.028837.

58. Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., and Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts

of the Arabidopsis thaliana seedling. Plant J. *50*, 660–677. https://doi.org/10.1111/j.1365-313X.2007.03078.x.

59. Czemmel, S., Stracke, R., Weisshaar, B., Cordon, N., Harris, N.N., Walker, A.R., Robinson, S.P., and Bogs, J. (2009). The grapevine R2R3-MYB transcription factor VvMYBF1 regulates flavonol synthesis in developing grape berries. Plant Physiol. *151*, 1513–1530. https://doi.org/10.1104/pp.109.142059.
60. Stracke, R., Jahns, O., Keck, M., Tohge, T., Niehaus, K., Fernie, A.R., and Weisshaar, B. (2010). Analysis of PRODUCTION OF flavonol glycosides-dependent flavonol glycoside accumulation in *Arabidopsis thaliana* plants reveals MYB11-, MYB12- and MYB111-independent flavonol glycoside accumulation. New Phytol. *188*, 985–1000. https://doi.org/10.1111/j.1469-8137.2010.03421.x.
61. Zhong, C., Tang, Y., Pang, B., Li, X., Yang, Y., Deng, J., Feng, C., Li, L., Ren, G., Wang, Y., et al. (2020). The R2R3-MYB transcription factor GhMYB1a regulates flavonol and anthocyanin accumulation in Gerbera hybrida. Hortic. Res. *7*, 78. https://doi.org/10.1038/s41438-020-0296-2.
62. Yuan, Y.-W., Byers, K.J., and Bradshaw, H.D. (2013). The genetic control of flower–pollinator specificity. Curr. Opin. Plant Biol. *16*, 422–428. https://doi.org/10.1016/j.pbi.2013.05.004.
63. Gehring, W.J., and Hiromi, Y. (1986). Homeotic genes and the homeobox. Annu. Rev. Genet. *20*, 147–173. https://doi.org/10.1146/annurev.ge.20.120186.001051.
64. Albert, N.W., Butelli, E., Moss, S.M.A., Piazza, P., Waite, C.N., Schwinn, K.E., Davies, K.M., and Martin, C. (2021). Discrete bHLH transcription factors play functionally overlapping roles in pigmentation patterning in flowers of *Antirrhinum majus*. New Phytol. *231*, 849–863. https://doi.org/10.1111/nph.17142.
65. Gegear, R.J., Burns, R., and Swoboda-Bhattarai, K.A. (2017). "Hummingbird" floral traits interact synergistically to discourage visitation by bumble bee foragers. Ecology *98*, 489–499. https://doi.org/10.1002/ecy.1661.
66. Orr, H.A. (2005). The genetic theory of adaptation: a brief history. Nat. Rev. Genet. *6*, 119–127. https://doi.org/10.1038/nrg1523.
67. Bower, C. (1996). Demonstration of pollinator-mediated reproductive isolation in sexually deceptive species of Chiloglottis (Orchidaceae: Caladeniinae). Aust. J. Bot. *44*, 15. https://doi.org/10.1071/BT9960015.
68. Moe, A.M., and Weiblen, G.D. (2012). Pollinator-mediated reproductive isolation among fig species (*Ficus*, Moraceae). Evolution *66*, 3710–3721. https://doi.org/10.1111/j.1558-5646.2012.01727.x.
69. Chittka, L., Shmida, A., Troje, N., and Menzel, R. (1994). Ultraviolet as a component of flower reflections, and the colour perception of hymenoptera. Vision Res. *34*, 1489–1508. https://doi.org/10.1016/0042-6989(94)90151-1.
70. Raguso, R.A., and Willis, M.A. (2002). Synergy between visual and olfactory cues in nectar feeding by naïve hawkmoths, Manduca sexta. Anim. Behav. *64*, 685–695. https://doi.org/10.1006/anbe.2002.4010.
71. Horth, L., Campbell, L., and Bray, R. (2014). Wild bees preferentially visit Rudbeckia flower heads with exaggerated ultraviolet absorbing floral guides. Biol. Open *3*, 221–230. https://doi.org/10.1242/bio.20146445.
72. Koski, M.H., and Ashman, T.-L. (2014). Dissecting pollinator responses to a ubiquitous ultraviolet floral pattern in the wild. Funct. Ecol. *28*, 868–877. https://doi.org/10.1111/1365-2435.12242.
73. Koski, M.H., and Ashman, T.-L. (2015). Floral pigmentation patterns provide an example of Gloger's rule in plants. Nat. Plants *1*, 14007. https://doi.org/10.1038/nplants.2014.7.
74. Koski, M.H., and Ashman, T.-L. (2015). An altitudinal cline in UV floral pattern corresponds with a behavioral change of a generalist pollinator assemblage. Ecology *96*, 3343–3353. https://doi.org/10.1890/15-0242.1.
75. Lunau, K., and Maier, E.J. (1995). Innate colour preferences of flower visitors. J. Comp. Physiol. A *177*, 1–9. https://doi.org/10.1007/BF00243394.
76. Briscoe, A.D., and Chittka, L. (2001). The evolution of color vision in insects. Annu. Rev. Entomol. *46*, 471–510. https://doi.org/10.1146/annurev.ento.46.1.471.
77. Chittka, L., Ings, T.C., and Raine, N.E. (2004). Chance and adaptation in the evolution of island bumblebee behaviour. Popul. Ecol. *46*, 243–251. https://doi.org/10.1007/s10144-004-0180-1.
78. Chittka, L., and Raine, N.E. (2006). Recognition of flowers by pollinators. Curr. Opin. Plant Biol. *9*, 428–435. https://doi.org/10.1016/j.pbi.2006.05.002.
79. Raine, N.E., Ings, T.C., Dornhaus, A., Saleh, N., and Chittka, L. (2006). Adaptation, genetic drift, pleiotropy, and history in the evolution of bee foraging behavior. Adv. Study Behav. *36*, 305–354. https://doi.org/10.1016/S0065-3454(06)36007-X.
80. Klahre, U., Gurba, A., Hermann, K., Saxenhofer, M., Bossolini, E., Guerin, P.M., and Kuhlemeier, C. (2011). Pollinator choice in petunia depends on two major genetic loci for floral scent production. Curr. Biol. *21*, 730–739. https://doi.org/10.1016/j.cub.2011.03.059.
81. Byers, K.J.R.P., Bradshaw, H.D., and Riffell, J.A. (2013). Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). J. Exp. Biol. *217*, 614–623. https://doi.org/10.1242/jeb.092213.
82. Fauser, F., Schiml, S., and Puchta, H. (2014). Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. Plant J. *79*, 348–359. https://doi.org/10.1111/tpj.12554.
83. Barbier de Reuille, P., Routier-Kierzkowska, A.-L., Kierzkowski, D., Bassel, G.W., Schüpbach, T., Tauriello, G., Bajpai, N., Strauss, S., Weber, A., Kiss, A., et al. (2015). MorphoGraphX: A platform for quantifying morphogenesis in 4D. eLife *4*, 05864. https://doi.org/10.7554/eLife.05864.
84. Concordet, J.-P., and Haeussler, M. (2018). CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. Nucleic Acids Res. *46*, W242–W245. https://doi.org/10.1093/nar/gky354.
85. R Core Team (2019). R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing).
86. Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.
87. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21. https://doi.org/10.1093/bioinformatics/bts635.
88. Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics *30*, 923–930. https://doi.org/10.1093/bioinformatics/btt656.
89. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550. https://doi.org/10.1186/s13059-014-0550-8.
90. Brandenburg, A., Kuhlemeier, C., and Bshary, R. (2012). Hawkmoth pollinators decrease seed set of a low-nectar *Petunia axillaris* line through reduced probing time. Curr. Biol *22*, 1635–1639. https://doi.org/10.1016/j.cub.2012.06.058.
91. Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis, Second Edition (Springer International Publishing). https://doi.org/10.1007/978-3-319-24277-4.
92. Harborne, J.B. (1998). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, Third Edition (Chapman and Hall).
93. Horsch, R.B., Fry, J.E., Hoffmann, N.L., Wallroth, M., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science *227*, 1229–1231. https://doi.org/10.1126/science.227.4691.1229.
94. Murray, M.G., and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res. *8*, 4321–4325. https://doi.org/10.1093/nar/8.19.4321.
95. Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucl. Acids Res. *19*, 1349. https://doi.org/10.1093/nar/19.6.1349.

96. Mallona, I., Lischewski, S., Weiss, J., Hause, B., and Egea-Cortines, M. (2010). Validation of reference genes for quantitative real-time PCR during leaf and flower development in Petunia hybrida. BMC Plant Biol. *10*, 4. https://doi.org/10.1186/1471-2229-10-4.

97. Bruce-White, C., and Shardlow, M. (2011). A Review of the Impact of Artificial Light on Invertebrates [Putting the Backbone into Invertebrate Conservation] (Buglife – the Invertebrate Conservation Trust).

98. Weaver, R.E. (2011). Effects of simulated moonlight on activity in the desert nightsnake (*Hypsiglena chlorophaea*). Northwest. Sci. *85*, 497–500. https://doi.org/10.3955/046.085.0308.

99. Yorzinski, J.L., Chisholm, S., Byerley, S.D., Coy, J.R., Aziz, A., Wolf, J.A., and Gnerlich, A.C. (2015). Artificial light pollution increases nocturnal vigilance in peahens. PeerJ *3*, e1174. https://doi.org/10.7717/peerj.1174.

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

CellPress

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Agrobacterium tumefaciens GV3101	Monsanto Company	N/A
Agrobacterium tumefaciens LBA4404	Monsanto Company	N/A
Escherichia coli DH5α	New England Biolabs	C2987H
Biological samples		
Petunia axillaris N	Rostock Botanical Garden (Germany)	N/A
Petunia axillaris P	University of Bern Botanical Garden	N/A
Petunia secreta	Universidade Federal de Minas Gerais, Belo Horizonte, Brazil	N/A
Chemicals, peptides, and recombinant proteins		
Q5 high fidelity DNA polymerase	New England Biolabs	Cat# M0491L
GoTaq DNA polymerase	Promega	Cat# M7845
$CaCl_2$	ThermoFisher	Cat# L13191.30
DNase I	Invitrogen	Cat# 18047019
KCl	ThermoFisher	Cat# 418205000
$MgSO_4$	ThermoFisher	Cat# 413485000
Boric acid	ThermoFisher	Cat# 033253.36
$CuSO_4$ x 5 H_2O	ThermoFisher	Cat# A11262.0I
Casein hydrolysate	ThermoFisher	Cat# J12855.P2
Sucrose	ThermoFisher	Cat# A15583.36
MES monohydrate	ThermoFisher	Cat# A16104.36
PEG 6000	ThermoFisher	Cat# J19972.A1
Muraschige-Skoog growth medium	Merck	Cat# M5519
Kanamycin	Merck	Cat# K1377
ORA SEE qPCR Green ROX L	HighQu	Cat# QPD0501
Biogluc	Andermatt Biocontrol AG	Cat# 2277C
Glucose monohydrate	ThermoFisher	Cat# A11090.36
Vitamin B1	Merck	Cat# 67-03-8
Vitamin B2	Merck	Cat# 83-88-5
Vitamin B6	Merck	Cat# 65-23-6
Folic acid	Merck	Cat# 59-30-3
Biotin	Merck	Cat# B4639
Critical commercial assays		
Gateway LR Clonase enzyme mix	ThermoFisher	Cat# 11791019
Gateway BP Clonase enzyme mix	ThermoFisher	Cat# 11789013
extraAXON plasmid mini kit	Axonlab	Cat# 11003321
innuPREP RNA Mini Kit 2.0	AnalytikJena	Cat# AJ 845-KS-2040050
qScriber cDNA synthesis kit	Axonlab	Cat# 11006057
TruSeq Stranded mRNA Library Kit	Illumina	Cat# 20020595
NovaSeq 6000 S Reagent Kit v1, 100 cycles	Illumina	Cat# 20012865
Deposited data		
P. axillaris N genome v3.03	BioProject	PRJNA689605
P. axillaris N myb-fl RNA-seq Illumina reads	BioProject	PRJNA894505

(*Continued on next page*)

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Bombus terrestris	Andermatt Biocontrol AG	Bombus Maxi Cat# 194C
Manduca sexta	Universität Kassel, Germany	N/A
Osmia cornuta	Naturschutzcenter Rottenburg, Germany	Cat# NSC14WDB004-1
Oligonucleotides		
A list of oligonucleotides is given in Table S7.	N/A	N/A
Recombinant DNA		
pDE-Cas9	Fauser et al.[82] (Addgene)	Cat# 61433
pDE-Cas9 myb-fl	This paper	N/A
Software and algorithms		
Geneious prime v2022.02.02	Biomatters	N/A
MorphoGraphX	Barbier de Reuille et al.[83]	N/A
CRISPOR	Concordet and Haeussler[84]	N/A
QuantStudio 5 Design and Analysis Software v1.4.3	Applied Biosystems	N/A
R v3.6.1	R Core Team[85]	N/A
Illumina bcl2fastq conversion software v2.20	Illumina	N/A
Trimmomatic v0.36	Bolger et al.[86]	N/A
STAR v2.6.0c	Dobin et al.[87]	N/A
featureCounts v1.5.2	Liao et al.[88]	N/A
DeSeq2 v1.26.0	Love et al.[89]	N/A
Maker v2.31.9	University of Utah, Department of Human Genetics	N/A
Other		
Resource website for this publication	This paper	https://github.com/Kuhlemeier-lab/single_gene_mutation_myb-fl_CB

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Cris Kuhlemeier (cris.kuhlemeier@ips.unibe.ch).

Materials availability

Plant lines generated in this study are available from the Institute of Plant Sciences, University of Bern, Switzerland (cris.kuhlemeier@ips.unibe.ch).

Data and code availability

- RNAseq data generated in this study have been deposited to SRA and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- All original code has been deposited on the Kuhlemeier GitHub repository and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- All software and algorithms are available from the references listed in the key resources table (section software and algorithms).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

P. axillaris N originates from the Rostock Botanical Garden in Germany and is registered in the VU Amsterdam collection as *P. axillaris* S26. *P. axillaris* P was obtained from the University of Bern Botanical Garden.[14] *P. secreta* was collected in its natural habitat Galpão de Pedra, Rio Grande do Sul, Brazil. A voucher is deposited at the herbarium of the Universidade Federal de Minas Gerais, Belo

Horizonte, Brazil. All plants were grown in commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand) under a light:dark regime of 15:9 h at 22:17 °C in a growth chamber. Plants were fertilized once a week with a nitrogen-phosphorous-potassium and iron fertilizer.

METHOD DETAILS

Color and UV image scoring

UV images were recorded with a converted Nikon D7000 SLR camera and Nikon 60 mm 2.8D microlens that blocks visible and infrared light to record UV light. Color images were recorded with a Canon EOS 60D camera and Canon 35 mm lens.

Measurement of additional floral traits

Corolla surface size and morphological traits (stamen and pistil lengths) were analyzed using front and side view photographs. Stamens were numbered according to their placement and size inside the floral tube (1 - 5 ranging from longest stamen to shortest in clockwise order around the floral axis); *Petunia* stamens are arranged in two different sizes (4+1).[35] For morphological traits, flowers were dissected to reveal reproductive organs inside the floral tube. All pictures were analyzed using the ImageJ software to extract lengths and areas of the samples. Three flowers per plant were analyzed as biological replicates. Nectar volume and concentration were measured according to the protocol described in Brandenburg et al.[90] All flowers were sampled right before the onset of dark in the growth chamber to account for any fluctuations in nectar production during the day:night cycle. Five flowers per plant were analyzed as biological replicates. Methylbenzoate, benzaldehyde and isoeugenol/eugenol compounds were analyzed as described previously in Amrad et al.[17] and Klahre et al.[80] by proton transfer reaction mass spectrometry (PTR-MS). Flowers were collected one day post anthesis right before the onset of dark in the growth chamber (15:9 h light:dark cycle at 22:17 °C). Scent volatiles of all plants were measured for 20 cycles. Five flowers per plant were analyzed as biological replicates. Isoeugenol and eugenol are isomers and can therefore not be separated via PTR-MS. They were thus analyzed as a single compound. Data was tested for normal distribution and statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons in R v3.6.1.[85] Results were considered significantly different if $p < 0.05$ and visualized using the ggplot2 package.[91]

Pollen germination measurements

Pollen germination rates were determined after incubation in germination medium. Germination medium consisted of a 10x salt solution (10 mM $CaCl_2$, 10 mM KCl, 8mM $MgSO_4$ x 7 H_2O, 16 mM boric acid, 300 μM $CuSO_4$ x 5 H_2O; salt solution end concentration 1x), 1.5% Casein hydrolysate (end concentration 0.03%), sucrose (final concentration 5%), MES monohydrate (end concentration 15 mM) and PEG 6000 (final concentration 12.5%). The media was adjusted to a pH value of 5.8 with 1 M KOH and filter sterilized. Single freshly dehisced anthers were dipped into 300 μl germination medium and incubated at room temperature for 2h. Care was taken to not shake samples after incubation to avoid damaging pollen tubes that had grown. Samples were prepared on microscopy slides after incubation and the percentage of germinated pollen grains was determined. Multiple groups were compared via a one-way ANOVA with Tukey *post hoc* comparisons in R v3.6.1[85] as data was normally distributed. Results were considered significantly different if $p < 0.05$ and visualized using the ggplot2 package in R.[91]

Quantification of flavonols and anthocyanins

Total flavonol and anthocyanin absorbance was measured for all flower petal limb samples using an Ultraspec 3100 pro (GE Healthcare Life Sciences) spectrophotometer, scanning from 365 nm to 530 nm. Three discs (8 mm diameter) were punched out of the floral limb tissue and placed in 1 ml of 2N HCl to soak for 15 min at room temperature (22 °C), hydrolyzed at 100 °C for 15 min, cooled for 10 min at 4°C, then centrifuged for 3 min at 14000 rpm. Supernatants were removed and analyzed directly to avoid degradation of pigments. Absorbance values for each sample were measured at 530 nm and 365 nm.[92] Five flowers were sampled per plant to include technical replicates for each biological replicate (individual plant). Results were visualized in R using the ggplot2 package.[91] For comparison of multiple samples statistics were calculated using a one-way ANOVA with Tukey *post hoc* comparisons after testing data for normal distribution in R v3.6.1.[85]

Confocal microscopy of epidermal cells

Epidermal peels were taken from the outer rim area of the petal limb of tested species and stained with 0.01% propidium iodide before placing them on microscopy slides for confocal microscopy imaging. Epidermal peels of 3 flowers of each species were taken for comparison. Images were taken with a Leica TCS SP5 microscope. To capture 3D images of the conical cells, we made z-stack images with 0.2 μM z-step size. Images were analyzed using ImageJ and MorphoGraphX.[83]

Generating CRISPR constructs

gRNAs targeting exons 3 and 4 of the *MYB-FL* gene were designed and checked for off-target effects using the CRISPOR tool.[84] Double gRNA constructs under the tomato U6 promoter and *A. thaliana* U6-26 promoter (Genbank X51447.1 and KY080693.1) containing attL sites for Gateway cloning were ordered from Genscript (New Jersey, USA). These constructs were then cloned into pDE-Cas9 destination vectors[82] using the LR reaction from the Gateway cloning protocol (Thermo Fisher). Final destination vectors were amplified in *E. coli* and then purified using the extraAxon plasmid mini kit (Axonlab). The destination vectors were

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

then transformed into *A. tumefaciens* strains GV3101 and LBA4404 and grown on solid selective medium at 28 °C for 2 days before being used for subsequent stable plant transformation. Multiple independent lines were carefully phenotyped.

Stable transformation of *P. axillaris*

P. axillaris N and *P. axillaris* P leaf discs were transformed essentially as described in Horsch et al.[93] using *A. tumefaciens* (strains GV3101 or LBA4404) carrying the respective CRISPR construct. Leaves from both *P. axillaris* species (from 4–6 week-old plants) were sterilized in a 10% hypochlorite solution for 10 min and rinsed five times with autoclaved MilliQ water. The sterilized leaves were then cut into 1 cm^2 pieces and inoculated in the *A. tumefaciens* suspension for 30 min (in the dark with shaking). After drying, the leaf fragments were transferred to Murashige-Skoog (MS) growth medium without antibiotic selection. Leaf pieces were grown in the dark for 7 days at a constant temperature of 24 °C. After this first week, leaf pieces were then transferred to fresh selective growth medium containing kanamycin to select for the presence of the CRISPR construct. All plates were sealed with medical tape and incubated at 24 °C and a light:dark regime of 16:8 h. Once shoots started to appear, each shoot was excised from the calli and transferred to MS rooting medium containing kanamycin for further selection of the construct.

DNA extractions and genotyping

Genomic DNA extractions were performed with fresh leaf samples using a modified CTAB[94] or modified SDS protocol.[95] Samples were analyzed with a Nanodrop ND-1000 (Thermo Fisher) prior to further analysis. Target sequences were amplified using the GoTaq (Promega) or Q5 polymerase (NEB) with primers depicted in Table S7.

RNA extractions, cDNA preparation and RT-qPCR

For each plant line collected, three individual buds (stage 4, 22-30 mm length) from four different plants grown under controlled conditions, were collected (n=12 for each sample type), representing biological replicates. The floral limb tissue of the buds was dissected and frozen immediately in liquid nitrogen. Other floral and plant organs were sampled under the same controlled conditions. For leaf samples, a single young fresh leaf was sampled, whereas all five sepals of an open flower were pooled for the sepal sample types. All stamens of an individual flower were also pooled for analysis while the pistil of the flower was sampled as a separate sample type. Tissue samples were stored at -80 °C until further processing. RNA extractions were performed using the innuPREP RNA Mini Kit 2.0 and cDNA was synthesized using the qScriber cDNA synthesis kit (Axonlab) and then used for RT-qPCR with the ORA SEE qPCR Green ROX L mix (Axonlab) according to the manufacturer's recommendations.

The expression levels of flavonoid pathway biosynthetic genes[30] were investigated by RT-qPCR. qPCR primers were published previously in Esfeld et al.[39] The reference genes used for all samples were *SAND*, *RAN1* and *ACTIN11*.[96] Primer efficiencies for each primer pair were determined using standard curves. RT-qPCR experiments were run on a QuantStudio 5, 384 well Real-Time PCR Machine (Applied Biosystems). All reactions were run in triplicate to calculate the means of the individual biological replicates using three data points. Using the QuantStudio Design and Analysis (v1.4.3) software the Cq values were determined for calculations. Values of the mutant and wildtype plants of each line were normalized to the three reference genes. For all analyzed biosynthetic genes pairwise comparisons with a Mann-Whitney U test were performed for each sample type of the mean relative expression levels in R v3.6.1[85] with results being significantly different if $p < 0.05$. The ggplot2 package was used to visualize the results.[91]

RNA sequencing

Quality of RNA was assessed using a Qubit 4.0 fluorometer (Thermo Fisher Scientific) and an Advanced Analytical Fragment Analyzer (Agilent). cDNA libraries were generated using a TruSeq Stranded mRNA Library Kit (Illumina) in combination with RNA UD Indexes (Illumina). The quality of the generated NGS libraries was evaluated using the same tools as described for RNA quality analysis. The pooled libraries were 100 bp single end sequenced using a NovaSeq 6000 SP Reagent Kit, v1, 100 cycles (Illumina) on an NovaSeq 6000 instrument (Illumina). All base call files were demultiplexed and converted into FASTQ files using Illumina bcl2fastq conversion software v2.20. The average number of reads/library was 41 million. The RNA quality-control assessments, generation of libraries and sequencing run were performed at the Next Generation Sequencing Platform, University of Bern, Switzerland.

Read processing and differential expression

The quality of the Illumina read data was processed using Trimmomatic v.0.36.[86] Processed reads were then mapped to the reference genome of *P. axillaris* N v.3.03 using STAR v.2.6.0c[87] in two-pass mode, with splice junctions –sjbdOverhang 100. Reads that mapped > 20 times were discarded. Reads were counted per gene using featureCounts v1.5.2.[88] Differential expression analysis was performed between mutant *P. axillaris* N *myb-fl* and *P. axillaris* N lines, and was performed with DESeq2 v.1.26.0[89] in R v3.6.1.[85] Read counts were normalized with rlog-transformation and sample replicates were used to compute mean counts per gene. Genes were considered significantly differentially expressed between the two lines if expression difference was at least 2-fold and *p-value* < 0.05. Functional annotations of differentially expressed genes were determined using the functional annotation scripts in Maker (v2.31.9) to call blastp (uniprot database accessed on 09/03/2020) and interproscan (v5.33.72.0). Interproscan additionally adds PFAM hits and assigns GO (gene ontology) terms.

Please cite this article in press as: Lüthi et al., Single gene mutation in a plant MYB transcription factor causes a major shift in pollinator preference, Current Biology (2022), https://doi.org/10.1016/j.cub.2022.11.006

Behavioral experiments

B. terrestris (bumblebee) colonies were obtained from Andermatt Biocontrol AG (Bombus Maxi; Grossdietwil, Switzerland) and maintained in the dark before experimentation (at 21 °C). Bumblebees were fed with pollen three times a week and were allowed to feed on a provided sugar solution (Biogluc, Biobest) at all times. The day before being used for behavioral assays, single bumblebees were extracted from the colony, given 500 μl of a 20% sucrose solution and isolated overnight in the dark. Assays were performed in a scent-saturated greenhouse during sunny conditions (greenhouse temperature 25 °C) in a flight cage with the following dimensions: 250 x 130 x 150 cm. Each bumblebee was presented with an array containing two plant species (or species and mutant) for choice experiments. Each array contained four plants per species, which were arranged in a 4 x 2 pattern with 30 cm distance between each plant. To control for differences in floral display, plants were manipulated to display 1 flower per plant. All flowers used for experiments were second day flowers post anthesis. Naive bumblebees were allowed to fly for 10 min each and all choices were recorded (see Figure S2A for types of encounters). If a bumblebee failed to fly or visit a flower during this time period, it was removed and not used for any further experiments (Table S6). All bumblebees were only used for a single choice assay.

The same experimental setup was also performed for a second type of bee pollinator: solitary bees *Osmia cornuta*. Pupae of *O. cornuta* were obtained from Naturschutzcenter (Rottenburg, Germany) in December and kept at 4° C until they were removed in spring prior to eclosion. Assays were performed as described for bumblebees in a flight cage with the following dimensions: 180 cm x 60 cm x 60 cm. Each array contained 2 plants per species, arranged in a 2 x 2 pattern with 20 cm distance between the plants. As half the number of plants were used, compared to the bumblebee setup, we controlled for floral display by allowing twice the number of flowers per plant (2 flowers per plant for long tube species; flowers second day post anthesis). Recording experiments was done as described for bumblebees (Figure S2A).

M. sexta female pupae were obtained from the group of Prof. Monika Stengl at the University of Kassel (Kassel, Germany). Pupae were reared in a climate chamber under controlled temperature and humidity with a light:dark regime of 16:8 h. After eclosion adult moths were fed with a nectar solution (protocol kindly provided by André Arand, Kassel, Germany) for two days. Nectar solution consisted of glucose monohydrate (33.72 g), fructose (27.68 g), sucrose (158.94 g), vitamin B1 (0.008 g), vitamin B2 (0.0036 g), vitamin B6 (0.0036 g), folic acid (0.0036 g) and biotin (0.00032 g) dissolved in 1L of water. Behavioral assays were conducted in a dark room with controlled temperature and humidity (21 °C, 60%) in a flight cage of 295 x 205 x 160 cm. Plants and moth growth chambers were synchronized to the same light:dark cycle to best mimic natural situations. Moths were transferred to the flight cage room 30 min prior to the start of experiments to acclimatize and initiate wing fanning. Low intensity lights emitting UV-A light as well as light in the visible blue range (spectral distribution 350 – 500 nm, average intensity 1 lux) was used during experiments to mimic lighting conditions during *M. sexta* natural flying conditions. Moonlight intensity has been calculated to be between 0.5 and 2 lux depending on the moon phase and lighting conditions.[97–99] Each moth was presented with an array containing two plant species for choice experiments. Each array contained four plants per species, which were arranged in a 4 x 2 pattern with 30 cm distance between each plant. To control for differences in floral display, plants were manipulated to display the same number of flowers per plant (1 flower per plant for long tube species). Naïve moths were observed for 10 min each and their encounters recorded (Figure S2B). All moths were used only once for a choice assay to eliminate any bias due to learning behavior.

Significant differences for all behavioral assays with pairwise comparisons of flower species were performed in R v3.6.1[85] using an exact binomial test. Results were considered significantly different if $p < 0.05$. Data was visualized using the ggplot2 package.[91]

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistics were calculated in R (v3.6.1). Statistical tests for the individual experiments as well as corrections for multiple comparisons are specific in the methods and figure legends. Results were considered significantly different if $p < 0.05$. All measured data are presented as means ± SD or SE and specified in the figure legends. Sample sizes (n) are also specified in the methods and figure legends.